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van der Zee, E.A.; Kronforst-Collins, M.A.; Maizels, E.T.; Hunzicker-Dunn, M.; Disterhoft, J.F.

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γIsoform-Selective Changes in PKC Immunoreactivity after Trace Eyeblink Conditioning in the Rabbit Hippocampus

E.A. Van der Zee,* M.A. Kronforst-Collins,
E.T. Maizels, M. Hunzicker-Dunn, and J.F. Disterhoft

Department of Cell and Molecular Biology, Northwestern University Medical School, Chicago, Illinois

ABSTRACT: An immunocytochemical examination of the rabbit hippocampus was done to determine which of the Ca^{2+}-dependent protein kinase C (PKC) isoforms (PKCα, -βI, -βII, or -γ) are involved in associative learning. The hippocampally dependent trace eyeblink conditioning task was used for behavioral training, and pseudoconditioned and naive animals served as controls. Significant increases ($P < 0.05$) in staining intensity were found with antibodies reactive with the catalytic or the regulatory domain of PKCγ in conditioned animals compared with naive and pseudoconditioned subjects at a 24-h post-conditioning time point. The increase was found in CA1 and CA3 pyramidal cell bodies, in apical dendrites and the proximal part of the basilar dendrites, and in cell bodies of dentate granule cells. In contrast, no conditioning-specific changes were found for PKCα, -βI, or -βII in hippocampal neurons. The increase in PKCγ immunoreactivity (ir) was significantly less ($P < 0.05$) in poor learners than in good learners. The correlation between the degree of PKCγ-ir and the total number of conditioned responses across training sessions was both positive and significant. These results suggest that PKCγ is the major Ca^{2+}-dependent PKC isoform involved in hippocampal neurons during acquisition of associative memories. Immunoblots revealed no conditioning-induced increase in the total amount or translocation of PKCγ at the 24-h time point, and no proteolytic PKC fragments were observed. In agreement with the Western blot data, PKC activity did not differ among naive, pseudoconditioned, and trace conditioned animals. The conditioning-induced increase in antibody binding to the γ-isoform must therefore be due to an increased access to the antigenic site(s) as a result of alteration in the tertiary structure of PKCγ or in quaternary interactions of PKCγ in situ. *Hippocampus 7:271–285, 1997.*

KEY WORDS: memory; associative learning; immunocytochemistry; Western blot

INTRODUCTION

Protein kinase C (PKC) is a cellular second messenger-regulated enzyme involved in various signal transduction pathways by which neurons increase their excitability in response to external inputs through phosphorylation of specific substrate proteins (Nishizuka, 1986, 1995). Prolonged activation of PKC, thought necessary for sustained cellular responses, can be realized through the enduring interaction of PKC with a variety of membrane lipid constituents (for review, see Nishizuka, 1995). Persistent changes in phosphorylation of PKC substrates that outlast an initial signal are most likely important molecular events in information storage. PKC activation, therefore, may serve as a critical step in the chain of biological events leading to memory formation.

Ample evidence in the last decade has demonstrated the involvement of PKC in learning and memory processes and long-term potentiation (LTP) (Akers et al., 1986; Bank et al., 1988; Olds et al., 1989, 1990; Wehner et al., 1990; Olds and Alkon, 1991; Scharenberg et al., 1991; Mathis et al., 1992; Paylor et al., 1992; Klann et al., 1993; Lucchi et al., 1993; Sacktor et al., 1993; Sunayashiki-Kusuzaki et al., 1993; Noguès et al., 1994; Golski et al., 1995). Initial studies (for a historical review, see Van der Zee and Douma, 1997) used the tritiated phorbol ester 12,13-dibutyrate ([^{3}H]PDBu) to probe PKC and to localize the changes (Bank et al., 1988; Olds et al., 1989, 1990; Olds and Alkon, 1991; Scharenberg et al., 1991; Golski et al., 1995). However, [^{3}H]PDBu has some important limitations: its specificity as a PKC probe has been questioned (Wilkinson and Hallam, 1994); it lacks isoform selectivity (Hug and Sarre, 1993; Dimitrijevic et al., 1995); and it has a relatively limited spatial resolution. These limitations can be circumvented by the use of isoform-specific PKC antibodies. Increased levels of immunoreactivity (ir) have been found in mouse and rat hippocampus after learning a spatial food retrieval task using a monoclonal antibody reactive with the regulatory domain of PKCγ (Beldhuis et al., 1992; Van der Zee et al., 1992, 1995b). The level of PKCγ-ir after spatial orientation correlated positively with the behavior in two rat strains (Luiten et al., 1994). Furthermore, region specificity of the changes in PKCγ-ir were determined by the type of learning task (Van der Zee et al., 1992, 1994a, 1995a,b).

The use of isoform-specific PKC antibodies should facilitate a more detailed identification of the neural

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*Correspondence to: E.A. Van der Zee, Department of Zoology, University of Groningen, Biologisch Centrum, Kerklaan 30, 9751 NN Haren, The Netherlands.
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substrate in which PKC plays a role. The goal of the present study, therefore, was to examine immunocytochemically which of the Ca^{2+}-dependent PKC isoforms (PKCα, -βI, -βII, and/or -γ) take part in the learning-specific alteration in PKC function in the hippocampus. Classical conditioning was used as a model system for studying the neural substrates of associative learning and memory (Disterhoft et al., 1996). Trace eyeblink conditioning, in which a stimulus-free "trace" period intervened between the tone (conditioned stimulus) and air puff (unconditioned stimulus), was used as a learning task because it is a more complex (higher order) form of conditioning than delay conditioning. It has been demonstrated to be dependent on the intact hippocampus for its successful acquisition and proper consolidation in the rabbit when subjects are trained with a sufficiently long trace interval (e.g., 500 ms) (Solomon et al., 1986; Moyer et al., 1990; Kim et al., 1995). Hippocampectomized rabbits show few conditioned responses during trace conditioning, which are inappropriately timed (Solomon et al., 1986; Moyer et al., 1990). These findings support the hypothesis that the hippocampus is involved in temporal information processing (Solomon, 1980; Rawlins, 1985). Besides its role in the acquisition of trace conditioning, the hippocampus seems to be involved in the temporary consolidation of the learned association as well. Lesioning of the hippocampus immediately after learning inhibited retention or reacquisition of trace eyeblink conditioning (Kim et al., 1995), whereas retention was intact when hippocampectomy was performed 30 days after acquisition, presumably after consolidation has occurred. Moreover, using hippocampal slices, Moyer et al. (1996) suggested that the time course of increased excitability of hippocampal pyramidal cells (lasting approximately 7 days) represents a critical window important for consolidation of the learned association elsewhere in the brain. Persistent alterations in the function of (some of the) Ca^{2+}-dependent PKC isoforms may underlie either the temporal information processing (the correct timing of the conditioned response) or the temporary information storage function of the hippocampus, or both. Parts of this study have been published in preliminary form elsewhere (Van der Zee et al., 1994b, 1995a).

MATERIALS AND METHODS

Animals

Forty-seven young adult New Zealand albino rabbits (*Oryctolagus cuniculus*, 2–3 months of age) were used in this study. The animals were housed individually under a light/dark cycle of 12:12 hour, with food and water available ad libitum. Animals were randomly assigned to one of three groups: naive (N; n = 13), pseudoconditioned (P; n = 17), or trace conditioned (T; n = 17).

Behavioral Training Procedure

Trace eyeblink conditioning trial presentation, data acquisition, and data analysis procedures were similar to those previously described (Moyer et al., 1990; Akase et al., 1994; Thompson et al., 1994). During behavioral training, rabbits were placed into cloth bags and then into Plexiglas restraining boxes. Subjects were trained in pairs in a sound attenuated chamber. The subjects were habituated to the apparatus for 60 min, at least 24 h prior to the first behavioral conditioning session.

The right eyelid was held open nonaversively with stainless steel clips to detect extension of the nictitating membrane. The trace eyeblink conditioning paradigm was used, in which a tone conditioned stimulus (CS) was followed by a 500-ms no-stimulus "trace" period, and then the air puff unconditioned stimulus (UCS). The CS was a 100-ms, 85-dB, 6-kHz tone presented through binaural headphones directly into the pinnae. The UCS was a 150-ms, 3-psi air puff directed at the cornea and was sufficient to elicit a reliable eyeblink response.

Training sessions consisted of 80 CS-UCS paired trials presented at a random intertrial interval ranging from 30 to 60 s and averaging 45 s. A conditioned response (CR) was defined as any blink that occurred after CS onset, but prior to UCS onset, and that met statistical criteria (>4 standard deviations above background mean level for ten successive 1-msec sampling bins). Rabbits were conditioned daily five times a week until a behavioral criterion of 80% CRs in an 80-trial session was reached, or for 15 training days in total. Slow learners were defined as rabbits that did not reach criterion within 15 training days, and who exhibited less than 40% CRs on the last training session. The slow and good learners were each pooled in a group. Although the behavioral performance of these two groups differed significantly from each other (see Results), the differentiation between good and slow learners is arbitrary; there is a continuum in the total number of CRs during training when all animals are pooled, as can be seen in Figure 5. Pseudoconditioned animals received explicitly unpaired presentations of the CS and UCS. The number of stimuli presented and the amount of time spent in the training chambers was identical to that received by conditioned subjects; only the association between stimuli was explicitly uncoupled. The pseudoconditioned controls were matched to the trace conditioned animals for direct comparison. The naive animals, which were neither habituated nor exposed to the tone or air puff stimuli, served as an extra control group (cage controls) providing the baseline values of PKC-ir. Subjects were killed 24 h after the last trial.

Immunocytochemical Procedure

Twenty-four hours after the last trial, a trace conditioned rabbit, its matched pseudoconditioned rabbit (matched in age and duration of handling and pseudotraining), and a naive rabbit were deeply anesthetized with a mixture of ketamine and xylazine and each transcardially perfused with 150 ml saline (40 ml/min) followed by 800 ml fixative composed of 2.5% paraformaldehyde, 0.05% glutaraldehyde, and 0.2% picric acid in 0.1 M phosphate buffer (PB) (pH 7.4). The brains were removed from the skull and cryoprotected by overnight storage in 30% sucrose in 0.1 M PB. Thereafter, immunostaining was carried out on frozen sections coronally cut at a thickness of 20 μm.

Free-floating sections were used to obtain a high-quality image of hippocampal PKC-ir for photography, while sections thaw-mounted on gelatin-coated slides were used for quantitative immunocytochemistry because their immunostaining was not saturated. In this case, sections of all three groups were collected on a glass slide to guarantee identical incubation conditions. Eight levels through the dorsal and posterior part of the hippocampus were collected, with an interval of approximately 500 μm. The range of the sampled hippocampal region corresponds approximately to level 53–66 in the brain atlas of Shek et al. (1986).

PKC isoforms were visualized by means of polyclonal rabbit IgG antibodies raised against peptides corresponding to C-terminal sequences contained in the catalytic domain of the α-, βI-, βII-, and γ-isoforms known to bind to mammalian PKC (C20, C16, C18, and C19, respectively; Santa Cruz Biotechnology, Santa Cruz, CA), and a monoclonal mouse IgG antibody, 36G9 (known to recognize rabbit PKCγ, Chemunex, Paris, France), raised against purified bovine PKCγ and reactive with the V2 region of the regulatory domain (Cazaubon et al., 1989, 1990). The tissue sections were preincubated for 15 min in 0.1% H_2O_2 in phosphate-buffered saline (PBS), subsequently rinsed in PBS, and immersed in 5% normal sheep serum (NSS) or normal goat serum (NGS) in PBS for 30 min for the polyclonal or monoclonal antibody, respectively, to reduce aspecific binding in the following incubation step. Next, the sections were incubated overnight at room temperature (RT) under gentle movement of the incubation medium containing the monoclonal antibody (36G9), diluted 1:200 in 1% NSS in PBS, or one of the polyclonal antibodies, diluted 1:200 in 1% NGS in PBS. After the primary incubation, sections were rinsed in PBS and again preincubated with 5% NSS or NGS for 30 min before the secondary incubation step in biotinylated sheep anti-mouse IgG (1:200; Amersham, Arlington Heights, IL) or biotinylated goat anti-rabbit IgG (1:200; Amersham), in PBS for 2 h at RT. Thereafter, the sections were thoroughly rinsed in PBS and incubated in streptavidin-horseradish peroxidase (Zymed, San Francisco, CA) diluted 1:200 in PBS for 2 h at RT. Finally, after subsequent rinsing in PBS and Tris buffer, the sections were processed by the diaminobenzidine (DAB)-H_2O_2 reaction (30 mg DAB and 0.01% H_2O_2/100 ml Tris buffer), guided by a visual check. Control experiments were performed by the omission of the primary antibody from the incubation medium, yielding immunonegative results. All photomicrographs of the DAB-processed material were taken under identical exposure conditions and coprocessed under similar printing conditions in the darkroom.

Tissue Fractionation for Western Blot Analysis and PKC Activity Assays

Four triplets of animals were used for both the Western blot analysis and the PKC activity assays. Conditioned animals were trained to the 80% criterion; matched pseudoconditioning and naive rabbits were used as controls as described above. Twenty-four hours later, animals were deeply anesthetized and decapitated. The brain was quickly removed from the skull and put on ice, and the hippocampi were rapidly dissected within 1.5–2 min. Blocks of the middle portion of the hippocampus (containing all subregions), weighing approximately 0.1–0.2 mg wet weight, were sampled. Blocks were each homogenized in a 1.0-ml volume of PPI buffer [protease/phosphatase inhibitor buffer, as described in Das et al. (1996)] containing (in mM) 80 β-glycerophosphate, 10 KPO_4, 1 EDTA, 5 EGTA, 10 $MgCl_2$, 2 dithiothreitol, 1 Na-ortho-vanadate, 100 μg/ml pepstatin A, 21 μg/ml leupeptin, 0.23 mM phenylmethylsulfonyl fluoride (PMSF), and 10% ethanol, pH 7.25. Homogenization was performed using 20–30 strokes in a hand-held ground-glass Potter Elvehjem homogenizer, and resulting homogenates were centrifuged for 70 min at 105,000× *g* (Hogeboom, 1955; Thomas et al., 1987) corresponding to 33,000 rpm in a Beckman SW 50.1 swinging bucket rotor in a Beckman model L8-70 preparative ultracentrifuge. The high-speed supernatants were collected for use as cytosol fractions (Hunzicker-Dunn and Jungman, 1978). The high-speed pellets, containing plasma membrane and pellet membrane-bound subcellular organelles, including nuclei, mitochondria, microsomes (Hunzicker-Dunn and Jungman, 1978), synaptosomes (Kikkawa et al., 1983), and cytoskeletal components (Kiley and Jaken, 1990), were resuspended by homogenization in 1.0 ml volumes (corresponding to the original homogenization buffer) of PPI buffer, and the resulting suspensions were used as pellet fractions. Aliquots of cytosol and pellet fractions respectively were prepared for subsequent Western blot analysis by the addition of Laemmli sodium dodecyl sulfate (SDS) sample buffer, followed by 5 min of heat denaturation at 100°C. Additional aliquots of cytosol and pellet fractions were collected for protein determination according to Lowry et al. (1951) using crystalline bovine serum albumin as a standard. Additionally, aliquots of cytosol and pellet fractions were stored at −70°C for subsequent PKC activity assays. Immediately prior to PKC activity assays, pellet fractions were subjected to resuspension by 10-s sonication.

Western Blotting Procedure

Cytosol and pellet fraction proteins were separated on SDS-polyacrylamide (8.5%) gels and then transferred to Nytran membrane (Schleicher & Schuell, Keene, NH). The blots were probed with anti-PKC isoform antibodies overnight at 4°C. [^{125}I]Protein A (~1 μCi/lane) was used to detect antigen-antibody complexes. Quantitation of bound I^{125} was done with the aid of an Imaging Densitometer (BioRad, model GS-670).

PKC Activity Assay

PKC activity was measured using a commercially available PKC assay kit, the Promega Signatect PKC assay kit (Promega, Madison WI), which is based on the use of neurogranin peptide $NG_{(28–43)}$ as a highly specific PKC substrate (Chen et al., 1993; Klann et al., 1993). The assay was performed as described in the manufacturer's protocol with minor modifications, as described below. PKC activity assay was performed on aliquots of cytosol and pellet fractions without further DEAE extraction (Chen et al., 1993). Phosphorylation of 5-μl fraction aliquots (containing 10 μg of cytosol protein or 25 μg of pellet protein) was carried out in

an incubation volume of 25 μl, containing 20 mM Tris-HCl buffer, pH 7.5, 0.2 mM EDTA, 1.0 mM EGTA, 12 mM $MgCl_2$, 0.1 mg/ml bovine serum albumin, 0.1 mM [γ-^{32}P]ATP (0.5 μCi/25 μl sample, specific activity of 4×10^2 cpm/pmol), and 100 μM biotinylated neurogranin peptide $NG_{(28–43)}$ substrate, with or without exogenous activators. Samples incubated with exogenous activators additionally contained 2.5 mM $CaCl_2$, 0.8 mg/ml phosphatidylserine, and 0.08 mg/ml diacylglycerol. Phosphorylation incubation was performed at 30°C for 5 min and was terminated by addition of 12.5 μl of 7.5 M guanidine-HCl termination buffer. Aliquots (10 μl) of the terminated reactions were spotted onto supplied streptavidin-matrix membrane, which was washed according to the manufacturer's protocol. Individual membrane squares were separated and counted for [^{32}P]phosphate incorporation by liquid scintillation spectrometry.

Phorbol Ester Application to Hippocampal Slices

Hippocampal slices were prepared from three young adult subjects according to the method described by Moyer et al. (1996). Animals were deeply anesthetized with halotane and quickly decapitated. The brain was removed from the skull and kept in ice-cold, oxygenated artificial cerebrospinal fluid (aCSF, composition in mM: 124 NaCl; 3 KCl; 1.3 $MgSO_4$; 1.24 NaH_2PO_4; 2.4 $CaCl_2$; 26 $NaHCO_3$; 10 d-glucose; gassed with 95% O_2-5% CO_2 at pH 7.4) for approximately 5 min before slices were prepared. The hippocampus from each hemisphere was dissected out over ice, and 4-mm-thick blocks were cut from the dorsal hippocampus. Tissue blocks were glued to a small, chilled chamber, which was then filled with ice-cold oxygenated aCSF. Hippocampal slices of 400 μm thickness cut on a Vibratome were maintained in a custom chamber filled with oxygenated aCSF. The slices were kept in this solution for 1 h before PDBu (Sigma) was added (1.0 μM). Slices were exposed to PDBu for 20 min and thereafter processed for either Western blotting (as described before) or immunostaining. For immunostaining, the slices were immersion fixed in 3% paraformaldehyde + 0.2% picric acid in 0.1 M PB for 45 min. After overnight storage in buffered 30% sucrose, frozen sections cut at a thickness of 20 μm were thaw-mounted on gelatin-coated slides and processed for immunocytochemistry (as described before).

Data Analysis

Mean learning curves (±SEM) and least-squares fitted lines are shown, expressed as the percentage of conditioned responses per block of 40 training trials. Using the linear interpolation algorithms of Igor (WaveMetrics, Lake Oswego, OR), each curve was normalized to the mean number of trials required to reach the criterion of 80% CRs.

Four immunostained brain sections of the dorsal and posterior hippocampus per animal were selected for each PKC isoform, and both the left and right hippocampi were analyzed for optical density (ODs). Analysis of the ODs described below were performed "blind" to the training condition of the animals. The ODs of PKC-ir of pyramidal cell bodies and their apical dendrites in the subiculum, CA1, CA3, and granule cell bodies in the dentate gyrus were measured. (These regions of interest are schematically delineated in Fig. 4C.) The average (±SEM) of the measured surface areas were: dentate granule cells (area 1: 0.19 $10^5 \pm 0.01\ 10^5$ mm^2); CA3 pyramidal cell bodies (area 2: 0.35 $10^5 \pm 0.03\ 10^5$ mm^2); apical dendrites of CA3 pyramidal cells (area 3: 0.33 $10^5 \pm 0.02\ 10^5$ mm^2); CA1 pyramidal cell bodies (area 4: 0.27 $10^5 \pm 0.02\ 10^5$ mm^2); apical dendrites of CA1 pyramidal cells (area 5: 0.28 $10^5 \pm 0.02\ 10^5$ mm^2); subicular pyramidal cell bodies (area 6: 0.33 $10^5 \pm 0.02\ 10^5$ mm^2); and apical dendrites of subicular pyramidal cells (area 7: 0.33 $10^5 \pm 0.02\ 10^5$ mm^2).

The OD was expressed in arbitrary units corresponding to gray levels using a Zeiss IBAS image analysis system. The value of background labeling was measured in the corpus callosum, which was relatively devoid of PKC-ir. The OD of the region of interest was related to the background value by the formula $[(OD_{area} - OD_{background})/OD_{background}]$, thus eliminating the variability in background staining among sections and correcting for different durations of the DAB reaction between triplets of animals.

Statistics

Analysis of variance followed by a post-hoc Student's *t*-test was used, with a probability level of $P < 0.05$ used as an index of statistical significance.

RESULTS

Behavioral Performance

The trace conditioned animals were divided in two groups (good and slow learners), based on their rate of acquisition (see Experimental Procedures). These animals showed a gradual increase in the percentage of trials on which CRs were exhibited, whereas the pseudoconditioned animals (P; n = 7) displayed a low and stable baseline throughout the experiment (Fig. 1). The slow learners (S; n = 6) showed a slow increase in the daily percentage CRs and reached approximately 40% CRs at day 15 (last day of training) (Fig. 1). The good learners (T; n = 7) reached the criterion of 80% CRs in a training session at day 10 (n = 2), day 12 (n = 2), or day 15 (n = 3). The rates of acquisition of the good learners were significantly faster than those of the slow learners ($P < 0.0001$); the rates of acquisition of the slow learners ($P < 0.05$) and of the good learners ($P < 0.00001$) differed significantly from those of the pseudoconditioned rabbits.

Analysis of PKC Isoform Immunoreactivity in the Hippocampus of Naive, Pseudoconditioned, and Trace Conditioned Rabbits

Immunostaining of the four different PKC isoforms had a predominantly postsynaptic appearance. It was found in the cell bodies and dendrites of the pyramidal cells (see Figs. 2 and 3 for the CA1 region), in dentate granule cell bodies (their dendrites, however, were only weakly stained), and in interneurons of the

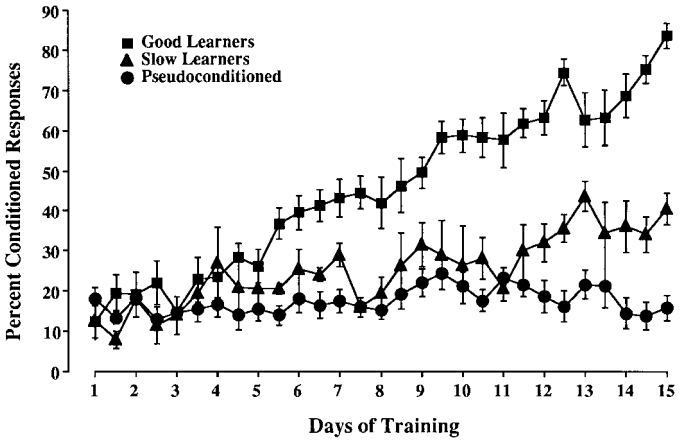

FIGURE 1. Learning curves expressed in percent conditioned responses (CRs) of trace conditioned animals, divided into good (n = 7) and slow (n = 6) learners. The slow learners averaged around 40% of CRs at the end of the experiment, while the trace conditioned animals reached the 80% criterion. Bars represent mean ± SEM.

stratum Oriens and Hilar region of the dentate gyrus (DG). The immunolocalization of the Ca^{2+}-dependent PKC isoforms is in good agreement with reports for the distribution and level of their mRNA (Young, 1988; Craig et al., 1993), and the distribution generally resembled that of rodents, with the exception of PKCβI. A high level of PKCβI mRNA was found in pyramidal cells of the rabbit hippocampus (Craig et al., 1993), and the level of ir we observed was also high. In rat hippocampus, the level of this protein is very low, and it is predominantly present in CA1 non-pyramidal cells (Hosoda et al., 1989; Saito et al., 1989). Our results showed that the level of PKCβI protein corresponded well with the level of its mRNA in the rabbit hippocampus.

In the dentate gyrus, interneurons immunoreactive for each of the isoforms could be seen. In the stratum oriens, the number of isoform-immunoreactive interneurons ranked as follows: PKCγ > PKCβI ≫ PKCα > PKCβII. Clear fiber staining was generally absent but could occasionally be seen for PKCα. Weakly immunoreactive astrocytes scattered throughout the hippocampus were found for all isoforms but were only rarely encountered with the 36G9 antibody for PKCγ. Alterations in PKC-ir in astrocytes in relation to trace eyeblink conditioning are dealt with in a separate paper (Van der Zee et al., 1996).

The optical densities (ODs) of the ir of all isoforms were measured in the pyramidal cell bodies and apical dendrites in the CA1 (see regions of interest 4 and 5 in Fig. 4C) of naive (N), pseudoconditioned and trace conditioned animals (good learners) (Figs. 2, 3). The distribution of PKC-ir in the thaw-mounted sections that were used for quantitation did not differ from that observed in free-floating sections. Large and significant ($P < 0.05$) increases in staining intensity were found for both antibodies reactive with the catalytic and regulatory domain of PKCγ in trace conditioned animals over naive and pseudoconditioned subjects. This effect was present in both the pyramidal cell bodies and their associated apical dendrites, as well as the proximal parts of the basilar dendrites of the CA1 and CA3 pyramidal cells (although these last were not analyzed in detail). The PKCγ-ir changes in the CA region were evenly distributed in most trace conditioned animals. However, in two animals (one slow learner and one good learner) enhanced PKCγ-ir in the CA1 region was seen in large patches, while the adjacent areas revealed a PKCγ staining intensity similar to that seen in naive or pseudoconditioned animals. All OD measures were taken from areas with even staining intensity. In contrast to PKCγ, no differences for PKCα and βII were observed between the groups, whereas the pseudoconditioned animals showed a significant increase ($P < 0.05$) for PKCβI in the pyramidal cell bodies only compared with naive and trace conditioned animals. No apparent changes in staining intensity were seen in interneurons of the stratum Oriens or Hilar region.

A more detailed analysis of the increase of PKCγ-ir as obtained by OD measures of the various hippocampal regions of interest (Fig. 4) was done using the monoclonal antibody 36G9 (because of the higher quality of immunostaining obtained with it than with the polyclonal C19). First, it was determined whether there was a difference between the left and right hippocampus. The OD values of the DG, CA3, CA1, and subiculum of trace conditioned animals were used to analyze possible lateralization (Table 1). No differences were found in the diverse hippocampal subregions between the left and right hemisphere ($P > 0.5$ in all cases). In Figure 4 (and Figs. 2 and 3), therefore, data from the left and right hippocampi were pooled. In addition to the groups presented in Figures 2 and 3, a group of six slow learners was added to the analysis. With the exception of the subiculum, the OD values of the different groups in the various hippocampal regions ranked as follows: T > S > P > N. The OD values showed a significant increase ($P < 0.05$) in PKCγ-ir in trace conditioned animals over naive and pseudoconditioned animals in the granule cells of the DG and in the cell bodies and apical dendrites of CA3 and CA1. The increase was most pronounced in the proximal part of the apical and basilar dendrites. The slow learners had intermediate OD values between the pseudoconditioned and trace conditioned subjects, but did not differ significantly from either group at any region of interest. However, the slow learners differed significantly from the good learners ($P = 0.027$), but not from the pseudoconditioned rabbits ($P = 0.409$), when the DG, CA3, and CA1 were taken together. No significant differences were found in the subiculum between the four groups ($P > 0.5$ for all comparisons). Neurons of the lateral hypothalamus, serving as a non-hippocampal and non-limbic system control region, showed no learning-specific changes. (OD values for the N, P, S, and T groups were 0.12 ± 0.02, 0.14 ± 0.04, 0.12 ± 0.03, and 0.13 ± 0.03, respectively.) In the forebrain, however, neocortical regions and some thalamic nuclei revealed enhanced PKCγ-ir to a similar extent as the hippocampal regions (data not shown), demonstrating that the changes were not solely restricted to the hippocampal formation.

The level of PKCγ-ir (averaged ODs of CA1 and CA3 pyramidal cell bodies and apical dendrites) and the total number of CRs across training sessions correlated positively for all trace conditioned animals (Fig. 5A; $r^2 = 0.761$; $P = 0.0002$). No correlation was found for the granule cells (Fig. 5B; $r^2 = 0.053$; $P = 0.4561$). The correlation for the CA1 and CA3 regions

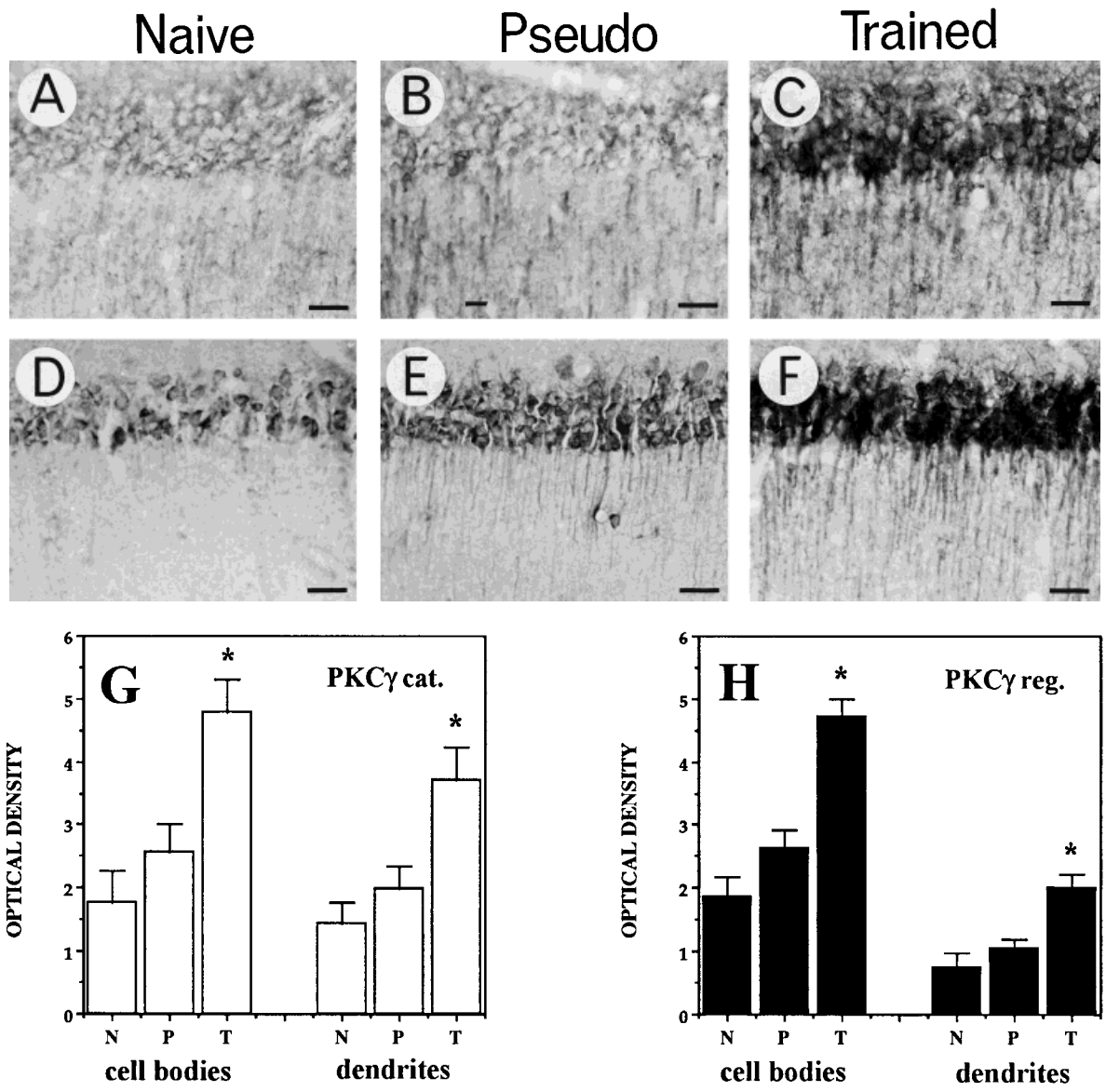

FIGURE 2. A–F: Photomicrographs of the hippocampal CA1 region of naive (Naive; A,D), pseudoconditioned (Pseudo; B,E), and trace conditioned animals (Trained; good learners, C,F) immunostained for the catalytic domain (D–F), or the regulatory domain of PKCγ (A–C). Optical densities of the immunostaining (G, catalytic domain; H, regulatory domain) were measured in the CA1 pyramidal cell bodies and associated apical dendrites (region of interest 4 and 5 in Fig. 4C). Bars indicate SEM. N, naive; P, pseudoconditioned; T, trace conditioned (good learners); *, significant increase ($P < 0.05$) over other groups. Scale bar in A–F = 25 μm.

separately was weaker than that of the averaged regions, suggesting that CA1 and CA3 pyramidal cells contribute in combination to control eyeblink responses at the behavioral level. The fact that the increase in PKCγ-ir relates to the acquisition of the behavioral conditioned response suggests that PKCγ may be an important molecular convergence point of the CS and UCS stimulus pathways in behavioral learning.

PKCγ Immunoblots of the Hippocampus From Naive, Pseudoconditioned, and Trace Conditioned Rabbits

Western blot analyses were performed to determine whether the increase in PKCγ-ir was due to: 1) the generation of the constitutively active catalytic fragment PKM of PKC; 2) an increase in the total amount of PKCγ; 3) translocation; or 4) a conformational change in PKCγ in situ, resulting in exposure of the antigenic site(s). Four triplets of animals were used, and the rate of acquisition of the four trace conditioned rabbits resembled that of the good learners (Fig. 1). The tissue blocks analyzed were taken from the dorsal and posterior part of the hippocampus and contained all subregions (i.e., DG, CA3, CA1, and subiculum). Figure 6A shows a typical immunoblot for PKCγ using 36G9 (regulatory domain). The immunoreactivity is seen at a migration position of approximately 80 kDa. No immunoreactive bands appeared around 30 kDa (for the single regulatory domain), indicating that proteolytic activation of PKCγ as a consequence of

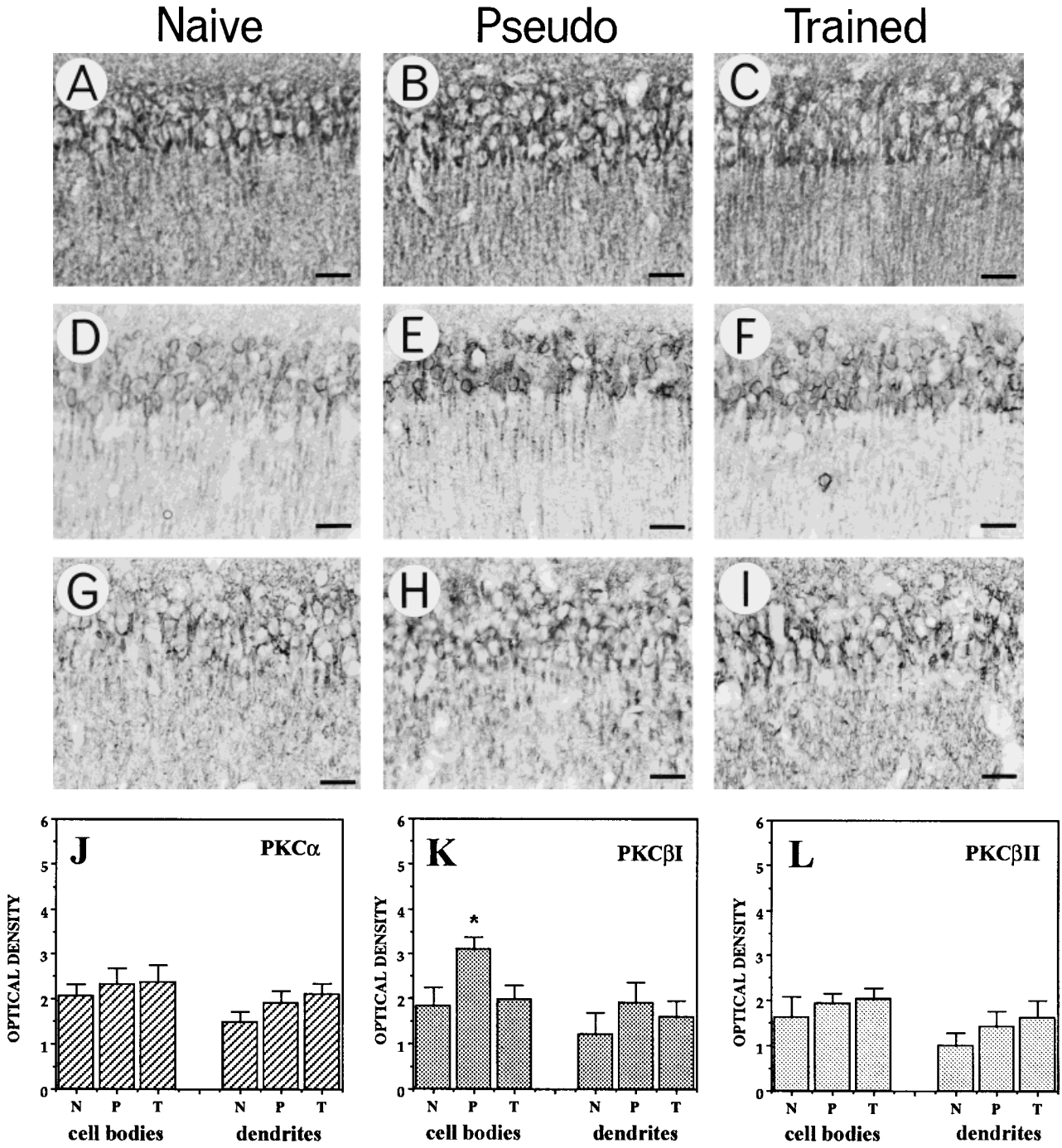

FIGURE 3. A–I: Photomicrographs of the hippocampal CA1 region of naive (Naive; A,D,G), pseudoconditioned (Pseudo; B,E,H), and trace conditioned animals (Trained; good learners, C,F,I) immunostained for PKCα (A–C), -βI (D–F), or -βII (G–I). Optical densities of the immunostaining for PKCα (J), -βI (K), and -βII (L) were measured in the CA1 pyramidal cell bodies and associated apical dendrites (region of interest 4 and 5 in Fig. 4C). Bars indicate SEM. N, naive; P, pseudoconditioned; T, trace conditioned (good learners); *, significant increase ($P < 0.05$) over other groups. Scale bar in A–I = 25 μm.

eyeblink conditioning was not the cause of the enhanced ir. Likewise, the absence of immunoreactive PKM fragments at 40–50 kDa on immunoblots performed with C19 (catalytic domain) supports this conclusion (data not shown). The ODs of the immunoreactive bands of the regulatory domain of PKCγ in the immunoblots were measured and depicted in Figure 6B. Results show that there are no differences between the groups, either in the total amount of PKCγ or in the amounts in the cytosol or pellet fractions. Similar results were found in the immunoblots for PKCα, -βI, -βII, and γ-catalytic (data not shown). After converting the ODs to the percent of PKCγ in the cytosol and pellet fraction, it became apparent that the γ isoform

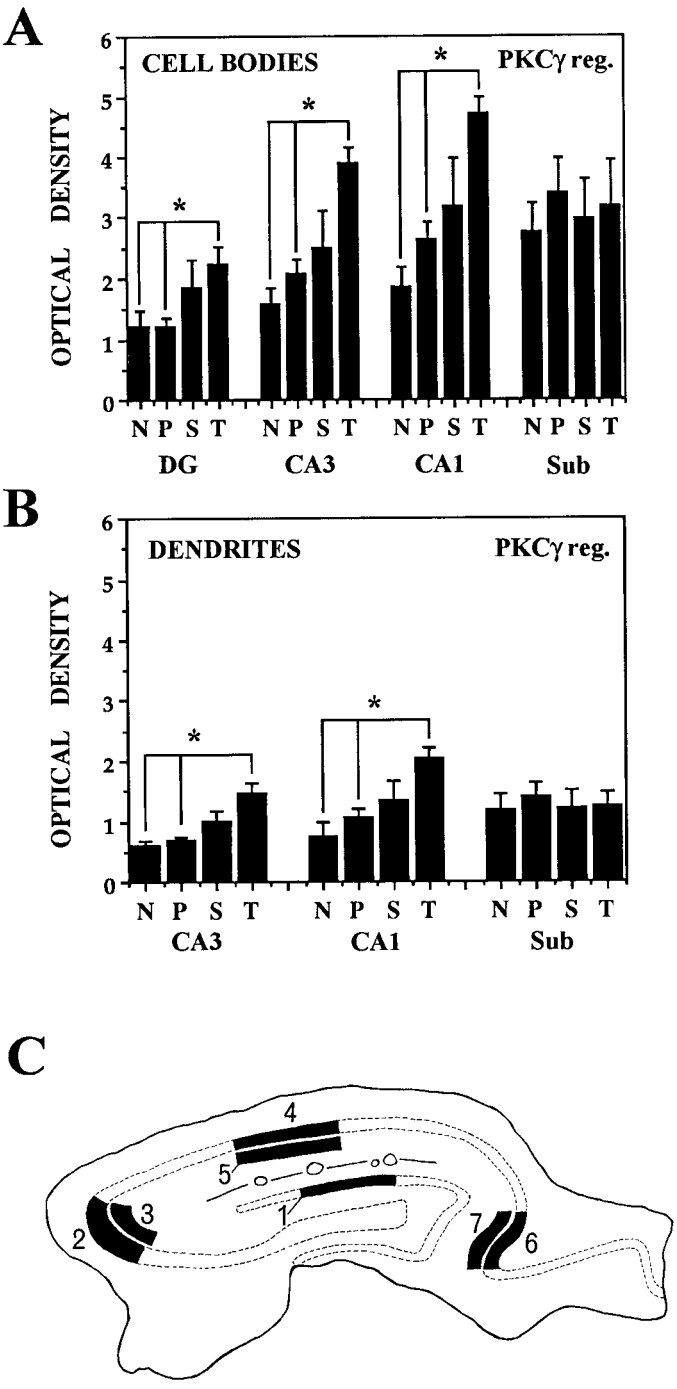

FIGURE 4. Optical densities of the immunostaining for the regulatory domain of PKCγ in cell bodies (A) and dendrites (B) in the dentate gyrus (DG), CA3, CA1, and subiculum (Sub). A schematic drawing (C) of the seven regions of interest (black blocks, numbered 1–7) in the hippo- campus at a characteristic level [level 56, approximately, according to the brain atlas of Shek et al. (1986)] shows the areas analyzed for optical density measures of PKC-isoform-ir. 1, granule cells of the inner blade of the DG; 2, CA3 pyramidal cell bodies; 3, apical dendrites of CA3 pyrami- dal cells in the stratum radiatum (predominantly corresponding to stratum lucidum); 4, CA1 pyramidal cell bodies; 5, apical dendrites of CA1 pyra- midal cells in the stratum radiatum; 6, subicular pyramidal cell bodies; 7, apical dendrites of subicular pyramidal cells. N, naive; P, pseudocondition- ed; S, trace conditioned (slow learners); T, trace conditioned (good learners); *, significant increase ($P < 0.05$) of T over N and P, but not S, in all cases.

was evenly distributed subcellularly between the soluble and pellet compartments in all groups (Fig. 6C), and that no translocation was seen at the 24 h post-conditioning time point. These results suggest that the conditioning-specific increase in PKCγ-ir observed at this time point is due to a conformational change of the molecule and the subsequent exposure of the antigenic site(s).

Trace Eyeblink Conditioning-Induced Enhancement of PKCγ-ir Is Mimicked in Hippocampal Slices by Phorbol Ester Application

Hippocampal slices were used to study whether the increase in PKCγ-ir could be experimentally mimicked by activating PKCs through the phorbol ester PDBu. Incubation (20 min) of hippocampal slices obtained from naive animals in a medium containing 1.0 μM PDBu resulted in a clear increase in the immunoreactivity for PKCγ compared with control slices similarly treated with the omission of PDBu from the incubation medium. All subregions of the hippocampal formation showed this increase in PKCγ-ir, including the subicular area. The OD measure of the immunostaining in slices revealed a significant ($P < 0.05$) increase in the CA1 pyramidal cell bodies and associated dendrites (Fig. 7A), resembling the differences seen between naive and trace conditioned animals (Fig. 2). Immunoblots of control and PDBu-treated slices (Fig. 7B) revealed that there was no generation of PKM, no increase in the total amount of PKCγ, and no significant translocation. (A translocation from the cytosol to the pellet fraction of 4.3% was found; Fig. 7C.) These findings were strikingly similar to those observed after trace eyeblink conditioning. In contrast to PKCγ, PKCα revealed a translocation from the cytosol to the pellet fraction of 17.1%, indicating that PKC can translocate under our experimental conditions.

PKC Activity Assay

PKC specific activity was determined in naive (n = 4), pseudoconditioned (n = 4), and trace conditioned (good learners; n = 4) animals. Cofactor stimulation of PKC resulted in a 3–5 times increase in activity compared with basal activity. As shown in Figure 8, however, no differences were found between the groups in the basal and cofactor-stimulated specific activity in either the cytosol or pellet fraction. Moreover, the total specific PKC activity (specific activity in cytosol and pellet combined according to the formula given in the legend of Table 2) and the percentage of PKC absolute activity in the cytosol and pellet fraction did not differ between the groups (Table 2).

DISCUSSION

PKCγ-Specific Alterations in Associative Memory Processes

The present results clearly demonstrate that PKC is involved in the sequence of molecular events that underlie associative learning

TABLE 1.

Left-Right Comparison of the OD Measures (±SEM) for PKCγ-ir in the Hippocampus of Trace Conditioned Rabbits[1]

	DG		CA3		CA1		Sub	
	L	R	L	R	L	R	L	R
Cell body	2.24 (±0.27)	2.20 (±0.33)	3.78 (±0.53)	3.95 (±0.50)	4.91 (±0.73)	4.51 (±0.62)	3.23 (±0.80)	3.12 (±0.80)
Dendrite	nd	nd	1.43 (±0.15)	1.47 (±0.18)	2.18 (±0.21)	1.86 (±0.26)	1.18 (±0.29)	1.28 (±0.26)

[1]No differences between the left (L) and right (R) hippocampus were found for any hippocampal subregion. DG, dentate gyrus; Sub, subiculum; nd, not determined (the PKCγ-ir was too weak for reliable OD measures).

and memory. Only PKCγ showed a consistent and robust increase in ir following hippocampally dependent trace eyeblink conditioning. These results and those of others provide evidence for isoform-specific functions and strongly point to brain-specific PKCγ as the primary isoform involved in learning and memory processes. Bowers et al. (1995) recently reported a selective strain difference for PKCγ-ir, but not for the -α, -βI, or -βII isoforms, in relation to the performance of hippocampally dependent learning tasks. Furthermore, translocation of PKCγ, but not PKCα or PKCβ, was observed following the induction of LTP in the rat DG, CA3, and CA1 (Angenstein et al., 1994).

PKCγ may form a critical interface between environmental stimuli and cellular processes occurring during learning and consolidation in pyramidal cells. The most likely location for such an interface function to occur is in the dendritic shaft and spines. Although all four Ca^{2+}-dependent PKC isoforms are present postsynaptically in dendritic shaft synapses, only the γ-isoform is nearly exclusively postsynaptic (Kose et al., 1990; Suzuki et al., 1993; but see the monkey hippocampus, Saito et al., 1994) and is abundantly present in dendritic spines (Kose et al., 1990; Tsujino et al., 1990; Saito et al., 1994). This subcellular localization of PKCγ makes it the most likely isoform candidate to be involved in learning and memory processes.

The changes for PKCγ-ir are region specific in the brain and subregion specific in the hippocampus (no changes were found in the subiculum). A major anatomical difference between the subiculum and the CA1 in the rabbit is the much less dense bundle of perforant path fibers innervating the subiculum, as can be seen in Nissl staining (Hjorth-Simonsen et al., 1975; Geinisman et al., 1996) or neurofilament immunostaining (Van der Zee et al., 1997). The rabbit subiculum (possibly in contrast to the rat; see Amaral and Witter, 1989; Lopes da Silva et al., 1990) does not, therefore, seem to receive such a massive input from the entorhinal cortex as do the DG, CA3, and CA1. It is likely that the excitatory input of the entorhinal cortex during conditioning trials triggers the increase of PKCγ-ir, possibly in concert with converging modulatory input from the cholinergic cells of the medial septum. An alternative explanation for the absence of changes in subicular PKCγ-ir could be that this region contains relatively high levels of PKCγ-ir in naive animals, which are either already maximal, or sufficient to perform the function necessary during trace eyeblink conditioning. Hippocampal slices of naive animals treated with PDBu, however, revealed a twofold increase in PKCγ-ir in all hippocampal areas (Fig. 7), including the subiculum. This finding demonstrates that the level of PKCγ-ir in this area can be enhanced and is not yet maximal.

The DG, CA1, and CA3 together exhibited a significantly lower level of PKCγ-ir in slow than good learners. A possible explanation for the difference in PKCγ-ir is that hippocampal neurons in the slow learners have a weaker receptor-PKCγ coupling system, which makes them less capable of becoming involved in the formation of the CS-UCS association at the molecular level. Alternatively, the neural systems that are engaged during trace eyeblink conditioning may activate this coupling system incrementally on each training trial in which a CR is produced. Since relatively fewer CRs were expressed in the slow learners, the total change in PKCγ-ir in their hippocampal neurons was smaller. The positive correlation found between the amount of PKCγ-ir observed and the total number of CRs given (Fig. 5) would support this possibility.

Like Sunayashiki-Kusuzaki et al. (1993), we found no translocation of PKCγ-ir at the 24-h post-conditioning time point. Moreover, no translocation of PKCγ was found (in contrast to PKCα) after phorbol ester treatment of hippocampal slices. Data regarding translocation of the γ-isoform of PKC is somewhat inconsistent throughout the literature and may largely depend on the experimental conditions. For example, phorbol ester treatment induced a very slow rate of translocation for PKCγ (Oda et al., 1991), and the degree of its translocation was lowest of all isoforms in the CA1 of hippocampal slices (Sacktor et al., 1993). It should be stressed, however, that PKC translocation, often found to be a relatively rapid and transient process (Nishizuka, 1995), could have occurred at earlier time points in the learning or consolidation process. Membrane-associated PKCγ could have been redistributed back to the cytosol by the 24-h post-conditioning time point. Moreover, the suggestion that prolonged translocation of PKC from the soluble to an integral membrane protein form plays an important role in memory processes (Alkon and Rasmussen, 1988; Burgoyne, 1989) has been questioned, based on the observation of rapid redistribution of membrane-

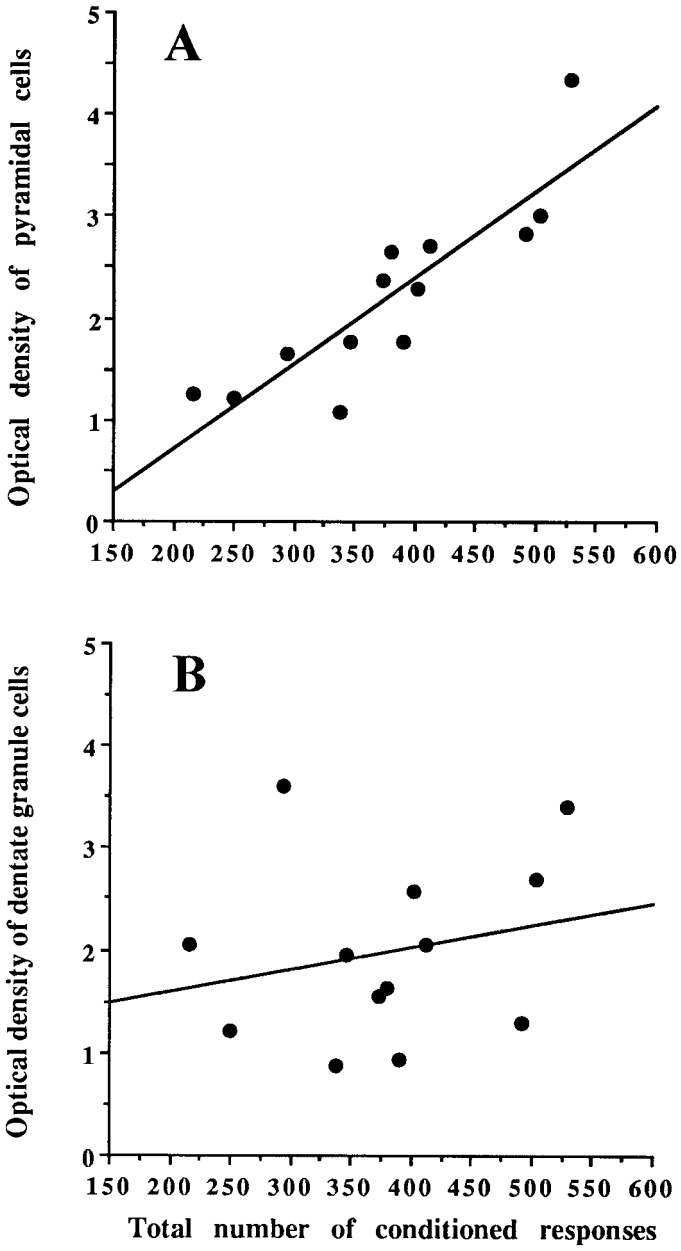

FIGURE 5. The optical density for PKCγ-ir in pyramidal cells correlated positively ($P = 0.0002$) with the total number of conditioned responses (A; the ODs of the CA1 and CA3 pyramidal cells are averaged), while no correlation ($P = 0.4561$) was found in dentate granule cells (B).

associated PKC to the cytosol after the dissociation of applied phorbol esters in vivo (Szallasi et al., 1994; Mosior and Newton, 1995).

The PKC activity assay corroborated the results from the Western blots by showing that no translocation of PKC was apparent at the 24-h post-conditioning time point. At this time

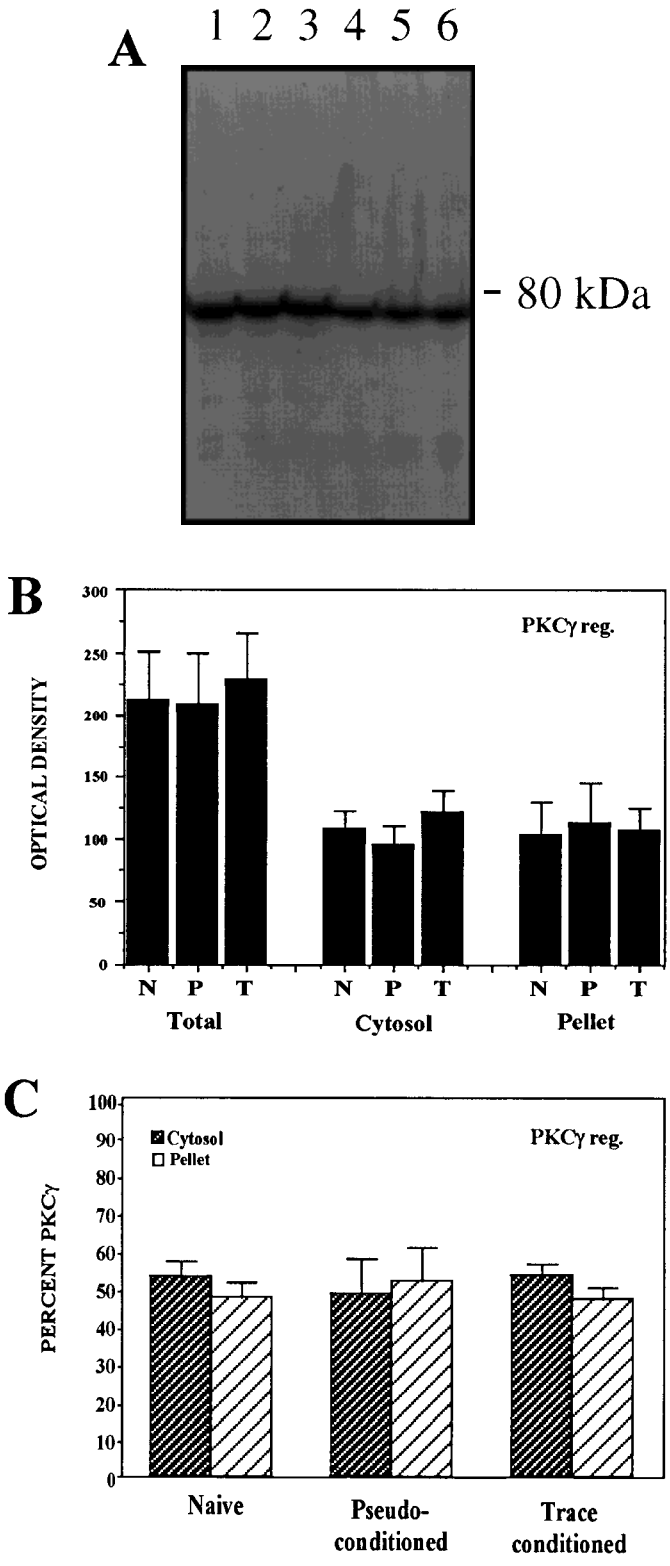

FIGURE 6. A: Example of an immunoblot for the regulatory domain of PKCγ of whole hippocampal tissue homogenates of naive (lanes 1 and 4, cytosol and pellet, respectively), pseudoconditioned (lanes 2 and 5, cytosol and pellet, respectively), and trace conditioned (good learners; lanes 3 and 6, cytosol and pellet, respectively) animals. Optical density measures of the immunoblots reveal no difference in the total amount (B) or in the subcellular distribution (C) of PKCγ between all three groups. N, naive (n = 4); P, pseudoconditioned (n = 4); T, trace conditioned (n = 4). Bars represent mean ± SEM.

point, PKC activity in trained animals was at baseline values. This does not rule out, however, that PKC activity had been different in trace conditioned animals at earlier times during the course of training.

Additional proposed mechanisms for promoting persistent activation of PKC in learning-related phenomena (notably LTP) include phosphorylation of PKC (Klann et al., 1993), oxidation of PKC (Palumbo et al., 1992), proteolytic cleavage (Suzuki et al., 1992; Sacktor et al., 1993), and altered membrane lipid concentrations resulting in a synergistic action of arachidonic acid and diacylglycerol (Lester et al., 1991; Bramham et al., 1994). It is unclear at present, however, which of these mechanisms contribute to the learning-related PKCγ changes presented here, although proteolytic cleavage can be ruled out. Besides the emphasis on (persistent) PKC activation and subsequent phosphorylation of PKC substrates as a key function in learning and memory processes, it should be noted that increased phosphorylation of these substrates is temporary (Ramakers et al., 1995). Dephosphorylation of PKC substrates, for example by calmodulin-dependent protein phosphatase (Seki et al., 1995), may prevent a prolonged training-induced phosphorylation state of PKC substrates. The role of dephosphorylation in learning and memory in relation to PKC activation therefore deserves further investigation.

Possible role of PKCγ in associative learning

Trace conditioning requires the formation of a short-term "memory-trace" of the CS (the tone) to bridge the interstimulus interval between the CS and UCS (the air puff) to form an association successfully and time the conditioned response correctly. The hippocampus is involved in the association of temporal events, and timing of the eyeblink response is crucial; it is suggested that in trace eyeblink conditioning the animal is likely to learn that "a tone followed by an empty interval means to blink" (Kim et al., 1995). Regulation of PKCγ activity and subsequent phosphorylation of PKCγ substrates in the hippocampus may be a key step underlying this function. The observed positive correlation of the level of PKCγ-ir and the number of correct behavioral CRs suggests that alterations in PKCγ are related to the temporary memory buffer function of the hippocampus to time the eyeblink response properly. A certain amount of previously immunonegative PKCγ may become available for binding of 36G9 and C19 in relation to each successfully timed eyeblink. This increase in PKCγ-ir could take place at one given set of synapses, at more and more synapses that are recruited as training continue, or at a combination of both. Hippocampal input corresponding to the CS and UCS pathways most likely stimulates receptors coupled to PKCγ. If the increase in PKCγ-ir

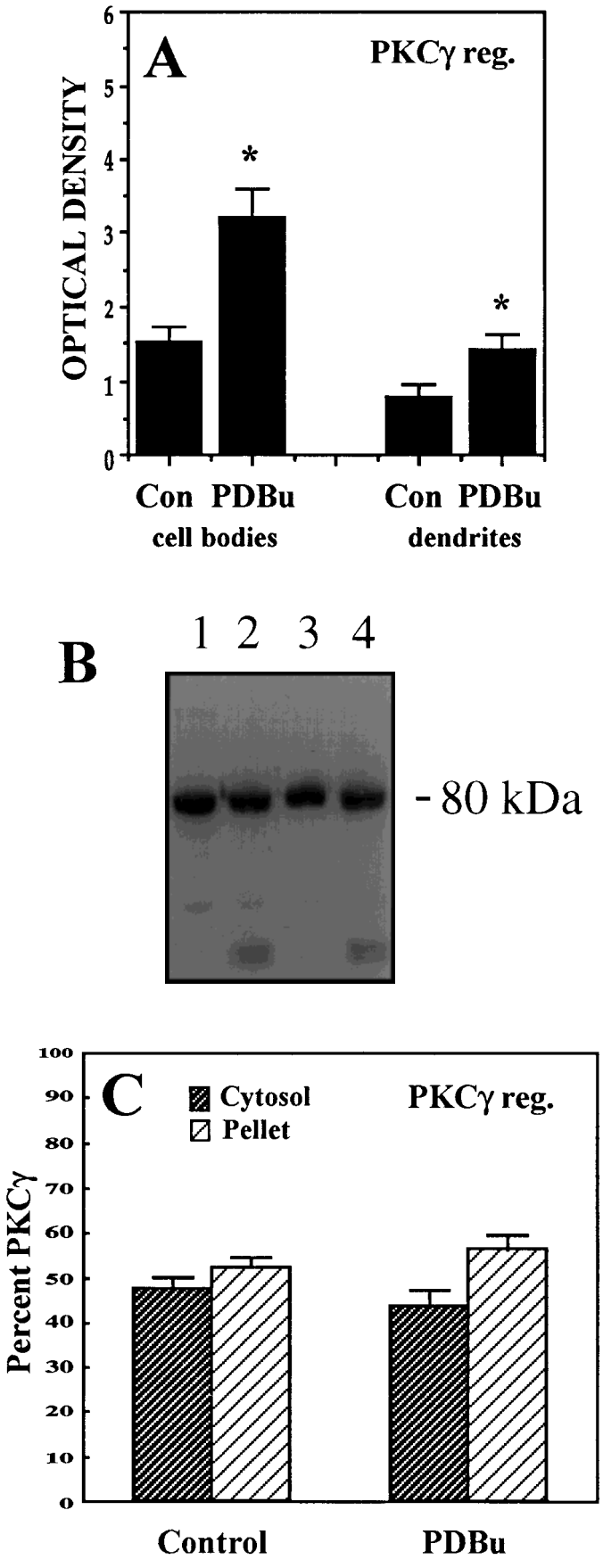

FIGURE 7. A: Optical density measures of PKCγ-immunostaining (regulatory domain) of sections taken from hippocampal slices show a significant ($P > 0.05$) increase in PKCγ-ir in pyramidal cell bodies and dendrites in phorbol ester (PDBu) treated over control (Con) slices. B: Representative immunoblot of γ-PKC of control and PDBu-treated slices. C: No significant difference was found in the subcellular distribution of PKCγ in the cytosol and pellet fractions. Bars represent mean ± SEM.

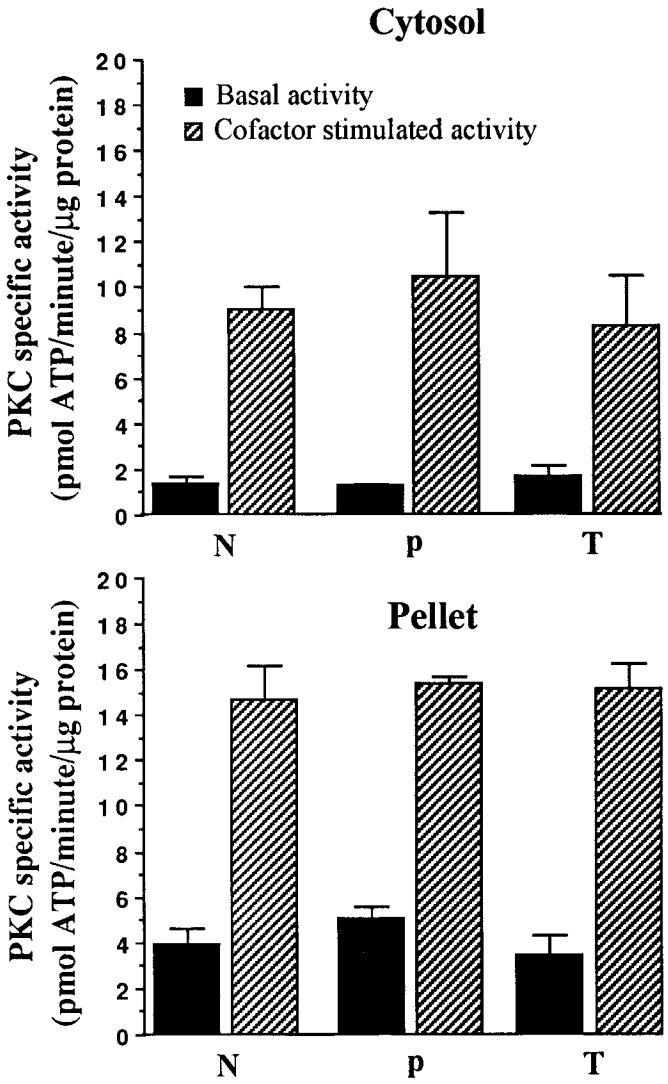

FIGURE 8. Basal and cofactor stimulated PKC specific activity in the cytosol and pellet fractions of naive (N; n = 4), pseudoconditioned (P; n = 4), and trace conditioned (T; n = 4) animals. The basal specific activity represents specific kinase activity in the presence of EGTA without Ca^{2+} or lipid activators. The cofactor-stimulated specific activity represents specific kinase activity in the presence of Ca^{2+} and lipid activators. For further details on the PKC activity assay conditions, see Materials and Methods. Bars represent mean ± SEM. No significant differences were found between the groups ($P > 0.05$ in all cases).

is a consequence of activation, it may have catalyzed the phosphorylation of specific substrate proteins in the synapse. For example, the afterhyperpolarization in hippocampal CA1 neurons, generated by a Ca^{2+}-activated potassium conductance (Hotson and Prince, 1980; Lancaster and Adams, 1986; Storm, 1990), is reduced after eyeblink conditioning (Disterhoft et al., 1986, 1988; Coulter et al., 1989; de Jonge et al., 1990; Moyer et al., 1996; Thompson et al., 1996). Phosphorylation of potassium channels through Ca^{2+}-dependent kinases and notably PKCγ in spines may be pivotal to the change in this conductance after conditioning (Etcheberrigaray et al., 1992). Other possible phosphorylation events may eventually result in enhanced synaptic strength, which seems crucial in (temporary) memory storage necessary for timing the behavioral response. In summary, the putative functional role of PKCγ in hippocampal principal neurons in trace eyeblink conditioning allows the animal to time the behavioral response correctly. A critical control experiment is delay conditioning, in which the hippocampus, although changed electrophysiologically and biochemically, is not critically involved. Consistent with our view of PKCγ functioning, hippocampal alterations in PKCγ-ir in fully delay eyeblink conditioned rabbits are limited and much less drastic than those seen in trace eyeblink conditioning (Van der Zee et al., 1995a).

A striking difference within the hippocampal formation was found between dentate granule cells and pyramidal cells. Pyramidal cells from trace conditioned rabbits revealed an increase in PKCγ-ir in both cell bodies and (apical) dendrites, whereas the dentate granule cells revealed a comparable increase in PKCγ-ir in their cell bodies only. A functional correlation for the difference in PKCγ-ir was found in both cell types as well; whereas the increase in PKCγ-ir correlated positively with the number of conditioned responses in pyramidal cells, no such correlation was found for the

TABLE 2.

PKC Activity Measures (±SEM) of Naive (n = 4), Pseudoconditioned (n = 4), and Trace Conditioned (n = 4) Animals

	Naive	Pseudo-conditioned	Trace conditioned
Total basal specific activity	3.30 ± 0.44	4.16 ± 0.47	2.93 ± 0.67
Total stimulated specific activity	10.04 ± 0.82	10.19 ± 0.58	10.76 ± 0.61
% Basal absolute activity			
Cytosol	8.39 ± 1.47	6.56 ± 0.99	11.80 ± 3.15
Pellet	91.61 ± 1.47	93.44 ± 0.99	88.20 ± 3.15
% Stimulated absolute activity			
Cytosol	16.66 ± 3.65	20.4 ± 7.42	12.91 ± 4.6
Pellet	83.34 ± 3.65	79.6 ± 7.42	87.09 ± 4.6

No differences were found between the various groups ($P > 0.05$ in all cases). The specific activity (SA) is expressed in units of pmol/min/μg protein. Total specific activity represents combined cytosol and pellet specific activities, calculated according to the following formula: $[(SA_{cytosol} \times \text{protein content}_{cytosol}) + (SA_{pellet} \times \text{protein content}_{pellet})]/[\text{protein content}_{cytosol} + \text{protein content}_{pellet}]$. The percentages of activity in the cytosol fraction are calculated as $(SA_{cytosol} \times \text{protein content}_{cytosol})/[(SA_{cytosol} \times \text{protein content}_{cytosol}) + (SA_{pellet} \times \text{protein content}_{pellet})] \times 100\%$.

granule cells. Interestingly, in contrast to pyramidal cells, no postburst after-hyperpolarization-reduction was observed in granule cells from trace conditioned rabbits in in vitro studies (de Jonge et al., 1990). Berger and Weisz (1987) summarized the different pattern of alteration observed in single units recorded from dentate granule cells and CA1 and CA3 pyramidal neurons during delay eyeblink conditioning. The granule cells showed an enhanced "sensory response" to the tone CS early in training, which did not increase as learning progressed. The CA1 and CA3 pyramidal neurons showed a gradually incrementing "modeling" of the developing conditioned response, which increased in correlation with the increase in size and probability of occurrence of eyeblink conditioned responses. The anatomical and functional differences for PKCγ among these cell types may contribute to the reported neurophysiological differences between the cell types.

Possible Mechanism Underlying the Increase in PKCγ-ir

Western blot analyses were performed to distinguish between mechanisms that might account for the observed increase in PKCγ-ir. Increased immunoreactivity can result from either: 1) an increase in the total amount of the antigen; or 2) increased access of the antibody to the antigenic site (Acevedo-Duncan et al., 1995). Results from Western blotting showed that there was no change in total amount of PKCγ as a result of conditioning, leading to the conclusion that the altered immunoreactivity must be a result of increased access of the antibody to the antigenic site. Increased access to the antigenic site consequent to translocation of PKC can be ruled out, since there was no alteration in the amount of PKC detected in either the cytosol or pellet fractions as a result of conditioning. Increased access to the antigenic site consequent to proteolytic processing of PKC can likewise be ruled out, since there were no detectable proteolytic fragments with either N-terminal or C-terminal-reactive antibodies. Increased access to the antigenic site must therefore result from alteration in the tertiary structure of PKCγ or in quaternary interactions of PKCγ in situ. A similar example of experimentally induced conformational change in PKC (PKCβ) that resulted in an increase in in situ immunostaining with no changes in Western blots is provided by Acevedo-Duncan et al. (1995). In this study, PKCβ-ir was significantly increased in human glioma cells after treatment with an antitumor drug, whereas Western blots showed no significant change in amount and no evidence of translocation of PKC. The equivalent increase in PKCγ-ir observed with the antibodies 36G9 and C19 indicates that the access of both antibodies was affected by conditioning. The finding that immunofluorescent double-labeling for 36G9 and C19 (data not shown) in hippocampal sections of conditioned animals revealed an increase in immunoreactivity for both antibodies at precisely the same subcellular locations of the pyramidal cell suggests that the increased immunolabeling takes place at the regulatory and catalytic domain of *the same individual* PKCγ molecules.

Our results suggest that the epitopes for 36G9 and C19 become exposed after activation of PKCγ. [Activation of PKCγ by PDBu in hippocampal slices mimics the increase in PKCγ-ir seen after associative learning (Fig. 7), as also previously demonstrated in mouse hippocampal tissue (Van der Zee et al., 1992)]. Sunayashiki-Kusuzaki et al. (1993) showed that associative learning potentiated subsequent PKC activation in the rabbit hippocampus. It is tempting, therefore, to speculate that the potentiated PKC relates to the immunopositive PKCγ in our study.

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